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ALTERED ACETYLCHOLINE RELEASE IN THE HIPPOCAMPUS OF DYSTROPHIN-DEFICIENT MICE

S. F. PARAMES,[a‡] E. D. COLETTA-YUDICE,[a†‡] F. M. NOGUEIRA,[a] M. B. NERING DE SOUSA,[a] M. A. HAYASHI,[a] M. T. R. LIMA-LANDMAN,[a] A. J. LAPA[a,b] AND C. SOUCCAR[a]*

[a] Department of Pharmacology, Section of Natural Products, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil

[b] Laboratory of Pharmacology and Toxicology, Amazon Biotechnology Center, Manaus, Amazonas, Brazil

Abstract—Mild cognitive impairments have been described in one-third of patients with Duchenne muscle dystrophy (DMD). DMD is characterized by progressive and irreversible muscle degeneration caused by mutations in the dystrophin gene and lack of the protein expression. Previously, we have reported altered concentrations of α7- and β2-containing nicotinic acetylcholine receptors (nAChRs) in hippocampal membranes of dystrophic (*mdx*) mice. This suggests that alterations in the central cholinergic synapses are associated with dystrophin deficiency. In this study, we examined the release of acetylcholine (ACh) and the level of the vesicular ACh transporter (VAChT) using synaptosomes isolated from brain regions that normally have a high density of dystrophin (cortex, hippocampus and cerebellum), in control and *mdx* mice at 4 and 12 months of age. ACh release evoked by nicotinic stimulation or K^+ depolarization was measured as the tritium outflow from superfused synaptosomes preloaded with [^{3}H]-choline. The results showed that the evoked tritium release was Ca^{2+}-dependent and mostly formed by [^{3}H]-ACh. β2-containing nAChRs were involved in agonist-evoked [^{3}H]-ACh release in control and *mdx* preparations. In hippocampal synaptosomes from 12-month-old *mdx* mice, nAChR-evoked [^{3}H]-ACh release increased by 57% compared to age-matched controls. Moreover, there was a 98% increase in [^{3}H]-ACh release compared to 4-month-old *mdx* mice. [^{3}H]-ACh release evoked by K^+ depolarization was not altered, while the VAChT protein level was decreased (19%) compared to that of age-matched controls. In cortical and cerebellar preparations, there was no difference in nAChR-evoked [^{3}H]-ACh release and VAChT levels between *mdx* and age-matched control groups. Our previous findings and the presynaptic alterations observed in the hippocampi of 12-month-old *mdx* mice indicate possible dysfunction of nicotinic cholinergic synapses associated with dystrophin deficiency. These changes may contribute to the cognitive and behavioral abnormalities described in dystrophic mice and patients with DMD.
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Key words: [^{3}H]-ACh release, nicotinic acetylcholine receptor, vesicular ACh transporter, hippocampus, dystrophin, *mdx* mouse.

INTRODUCTION

Varying degrees of cognitive impairment and behavioral disorders have been associated with dystrophin deficiency in patients with Duchenne muscle dystrophy (DMD) (Mehler, 2000; D'Angelo and Bresolin, 2006). DMD is an X-linked muscle disease caused by mutations in the gene that encodes dystrophin resulting in a dysfunctional protein or lack of the protein itself (Hoffman et al., 1987). The myopathy is highly incident in boys and is characterized by progressive and irreversible muscle degeneration (Blake et al., 2002). In contrast, the cognitive deficits of DMD patients are non-progressive and unrelated to the severity of muscle wasting (Cyrulnik et al., 2008; Hendriksen and Vles, 2008; Snow et al., 2013).

Dystrophin is a large protein (427 kDa) expressed in muscles and neuronal cells, localized on the inner face of the plasma cell membrane. This protein connects the intracellular γ-actin to the extracellular laminin through a transmembrane protein complex, the dystrophin-associated glycoprotein complex (DAPC) (Ervasti, 2007; Waite et al., 2009). In striated muscles, the DAPC provides structural stability to the sarcolemma during muscle contraction (Lapidos et al., 2004). Dystrophin and the associated protein complex are also expressed in postsynaptic regions of the neuromuscular junction. Here, they are involved in the maturation of the postjunctional folds and the stabilization of the nicotinic

*Corresponding author. Address: Department of Pharmacology, Section of Natural Products, Escola Paulista de Medicina, Universidade Federal de São Paulo, 04044-020 Rua Três de Maio 100, São Paulo, SP, Brazil. Tel: +55-11-5576-4447; fax: +55-11-5576-4499. E-mail address: csouccar@unifesp.br (C. Souccar).

† Present address: Faculdades Oswaldo Cruz, São Paulo, SP, Brazil.

‡ These authors contributed equally to this work.

Abbreviations: [^{125}I]-αBGT, [^{125}I]-α-bungarotoxin; ACh, acetylcholine; AChE, acetylcholinesterase; ANOVA, analysis of variance; ATR, atropine; CA1, cornus ammonis 1 of hippocampus; DAPC, dystrophin-associated glycoprotein complex; DHBE, dihydro-β-erythroidine; DMD, Duchenne muscle dystrophy; ECL, enhanced chemiluminescence; EGTA, ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); KB buffer, Krebs-bicarbonate buffer; Ki, inhibition dissociation constant; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline and Tween-20; Tris–HCl, tris (hydroxymethyl) aminomethane hydrochloride; VAChT, vesicular acetylcholine transporter; VGCCs, voltage-gated Ca^{2+} channels.

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acetylcholine receptor (nAChR) clusters (Grady et al., 2000; Huh and Fuhrer, 2002; Ghedini et al., 2008). Two other full-length isoforms of dystrophin, regulated by different promoters are expressed predominantly in the cortical and hippocampal pyramidal neurons, in the cerebellar Purkinje cells and in the amygdala (Lidov et al., 1990; Blake et al., 2002; Sekiguchi et al., 2009). In these brain regions dystrophin is found at postsynaptic densities of inhibitory neuronal synapses (Kim et al., 1992; Lidov et al., 1993; Sakamoto et al., 2008) where the protein is co-localized with a subset of $GABA_A$ receptor ($GABA_AR$) clusters (Knuesel et al., 1999; Sekiguchi et al., 2009). The lack of the full dystrophin isoform in the *mdx* mouse model has been correlated with a decrease in the number of $GABA_AR$ clusters in the hippocampus, cerebellum (Knuesel et al., 1999; Kueh et al., 2008) and amygdala (Sekiguchi et al., 2009). Dystrophin-deficient mice also show a significant decrease in the density of kainate-type glutamate receptors (Yoshihara et al., 2003). These studies suggest that dystrophin is involved in anchoring and stabilizing receptors and ionic channels in neuronal cells, which indicates the possibility of a role in synaptic function (Perronnet and Vaillend, 2010; Pilgram et al., 2010).

Previous binding studies conducted by our group, using membrane preparations and specific ligands for $\alpha4\beta2$ ([^{3}H]-cytisine) and $\alpha7$ ([^{125}I]-α-bungarotoxin ([^{125}I]-αBGT)) nAChRs subtypes, have shown a significant increase of the maximum number (Bmax) of [^{125}I]-αBGT binding sites, in the hippocampal membranes of 4-month-old *mdx* mice as compared to age-matched controls. In contrast, Bmax values for both [^{3}H]-cytisine and [^{125}I]-αBGT binding sites had decreased in older (12-months) *mdx* mice compared to those of age-matched controls. There were no alterations in the receptors affinity (Kd) to the ligands in either case. Likewise, there were no changes in the mRNA transcripts for the $\alpha4$, $\alpha7$ and $\beta2$ nAChR subunits in the hippocampus, indicating that post-translational mechanisms were involved (Ghedini et al., 2012). However, Wallis et al. (2004) reported a decrease in the expression of the $\alpha3$ nAChR subunit mRNA in the cortex and hippocampus of young (<4 months) *mdx* mice as compared to age-matched controls. In contrast, the density of muscarinic ACh receptors did not differ between control and *mdx* mice (Yoshihara et al., 2003). These observations suggest possible alterations in nicotinic synaptic function in the hippocampus, a brain region involved in attention, memory and learning functions (Kenney and Gould, 2008; Drever et al., 2011), which may be related to the cognitive impairments reported in DMD patients (Cyrulnik et al., 2008; Snow et al., 2013).

nAChRs are ligand-gated cationic channels that mediate transmission in the peripheral (neuromuscular junction, autonomic ganglia) as well as the central nervous system (CNS). In the mammalian brain, these receptors are involved in cognitive functions and have been implicated in neurological and psychiatric disorders such as Alzheimer's disease, Parkinson's disease, attention-deficit hyperactivity disorder, schizophrenia, anxiety and depression (Sacco et al., 2004; Levin et al., 2006; Hurst et al., 2013). Neuronal nAChRs consist of a combination of different α ($\alpha2$–$\alpha6$) and β ($\beta2$–$\beta4$) subunits that form heteropentamers, or they may be comprised of a single α ($\alpha7$, $\alpha8$ and $\alpha9$) subunit forming homopentamers (Albuquerque et al., 2009; Millar and Gotti, 2009). Two nAChR subtypes are predominantly found in the brain: the $\alpha4\beta2^*$ subtype (*indicate possible additional subunits, Millar and Gotti, 2009), which is characterized by a high-binding affinity to epibatidine and is insensitive to α-BGT, and the $\alpha7^*$ subtype, which has a low-binding affinity to epibatidine, is sensitive to α-BGT and exhibits higher permeability to Ca^{2+} than the $\alpha4\beta2^*$ subtype (Fucile, 2004; Marks et al., 2010). Despite their involvement in mediating rapid synaptic transmission at cholinergic synapses, the main role of nAChRs in the CNS is to regulate the release of various neurotransmitters. nAChRs localized on presynaptic and perisynaptic regions may regulate the release of ACh (autoreceptors) and of different neurotransmitters (heteroreceptors) such as glutamate, GABA, dopamine, noradrenaline and serotonin (Wonnacott, 1997; MacDermott et al., 1999; Picciotto et al., 2012).

To investigate the influence of dystrophin on central cholinergic synaptic function, this study aimed to examine the release of [^{3}H]-ACh evoked by nAChRs activation and the level of the vesicular ACh transporter (VAChT), a cholinergic marker, in brain structures that normally express dystrophin in mice. The analyses were performed using synaptosomes and synaptic vesicle fractions isolated from the cerebral cortex, hippocampus and the cerebellum of the same control and *mdx* mice groups used in our previous study, at 4 and 12 months of age (Ghedini et al., 2012). The 12-month-old animal group was included because behavioral and biochemical abnormalities in the brain were seen in *mdx* mice older than 6 months (Rae et al., 2002).

EXPERIMENTAL PROCEDURES

Animals

Control (typically developing) and mutant *mdx* male mice from the C57Bl/10 strain bred in the Animal Facility at Instituto Nacional de Farmacologia e Biologia Molecular (INFAR), Escola Paulista de Medicina/Universidade Federal de São Paulo (EPM/UNIFESP) were used at the age of 4 and 12 months. All animals were housed under a controlled 12/12-h light/dark cycle at 22 ± 2 °C with free access to food and water. The brain was removed immediately after animal decapitation between 9:00 and 10:00 a.m., and the regions of interest were dissected over a cold plate. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the USA National Institutes of Health (Bethesda, Maryland). All the procedures were approved by the institutional Animal Investigation Ethics Committee (Protocol N° 1178/10). Every effort was made to minimize the number of animals used and their suffering.

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